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Mechanisms of regulation and diversification of Deubiquitylating enzyme function

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Abstract

Deubiquitylating enzymes (DUBs) are proteases that reverse protein ubiquitylation and therefore modulate the outcome of this post-translational modification. DUBs regulate a variety of intracellular processes, including protein turnover, signalling pathways and DNA damage response. They have also been linked to a number of human diseases, such as cancer, inflammatory and neurodegenerative disorders. Whilst we are beginning to better appreciate the role of DUBs in basic cell biology and their importance for human health, there are still many unknowns. Central amongst these is a conundrum of how a small number of approximately 100 DUBs encoded in the human genome is capable of regulating the thousands of ubiquitin modification sites detected at steady-state conditions in human cells. This Commentary addresses the biological mechanisms employed to modulate and expand the functions of DUBs, and sets directions for future research aimed at elucidating the details of these fascinating processes.

Introduction

Post-translational attachment of ubiquitin, or ubiquitylation, controls most intracellular processes, including protein turnover, intracellular signalling, endocytosis and DNA damage response (Clague and Urbe, 2017; Heideker and Wertz, 2015; Kee and Huang, 2015). Ubiquitin is conjugated most often to lysine residues of target proteins in a reaction catalysed by an enzymatic cascade that involves the E1 activating, E2 conjugating and E3 ligating enzymes. Importantly, ubiquitin can also be ligated to any of the seven internal lysine residues or the N-terminal methionine of another ubiquitin forming chains whose linkage type defines the outcome of protein ubiquitylation. Ubiquitylation can be reversed by deubiquitylating enzymes (DUBs) that cleave ubiquitin off the substrate protein or within ubiquitins in a polyubiquitin chain (Fig. 1). The importance of the components of the ubiquitin conjugation and deconjugation systems is underscored by the fact that their deregulation has been linked to the pathogenesis of a number of human diseases, such as cancer, neurodegenerative, inflammatory and metabolic disorders (Heideker and Wertz, 2015).

Approximately 100 DUBs are encoded in the human genome (Nijman et al., 2005b). Depending on the organisation of the catalytic domain, DUBs can be classified into distinct families (Table 1), a vast majority of which are cysteine proteases. These include ubiquitin-specific proteases (USPs), ubiquitin C-terminal hydrolases (UCHs), ovarian tumour proteases (OTUs), Machado-Joseph disease proteases (MJDs) and the recently discovered (Abdul Rehman et al., 2016) MIU-containing novel DUB family (MINDY) proteases. Additionally, there is also a metalloprotease family of DUBs, the JAB1/MPN/Mov34 (JAMM) domain proteases (Nijman et al., 2005b). Strikingly, although DUBs have been implicated in many cellular processes, in most cases their precise function is still either poorly characterised or completely unknown. Importantly, there are at least 600 E3 ubiquitin ligases, and at steady-state conditions, approximately 20,000 ubiquitylation sites on thousands of intracellular proteins can be detected (Clague et al., 2015; Kim et al., 2011; Udeshi et al., 2013). How a relatively small number of DUBs manages to regulate such a vast number of modifications is one of the key unsolved questions in the field. In this Commentary, we will discuss the different layers of regulation of DUB activity that ensure that DUBs are activated, regulated and targeted to their appropriate substrates to achieve precise spatio-temporal control of

ubiquitin signals within a specific physiological pathway. We will discuss these concepts with an emphasis on how functional diversification of DUBs can be achieved.

Regulation of DUB abundance

Conceptually, the simplest mechanism for modulating the activity, and hence biological function, of a given protein is by regulating its intracellular concentration. This also applies to DUBs whose abundance can be controlled both via transcription or translation and degradation. For example, levels of several DUBs are regulated in a cell cycle-dependent manner and are directly related to their function. USP37 is one such DUB, and its transcription is controlled by the E2F transcription factors, resulting in high USP37 levels during G1/S phase transition (Huang et al., 2011). This in turn allows for the stabilisation of cyclin A and cell cycle progression into S phase. Interestingly, USP37 is degraded in mitosis by the anaphase promoting complex APC^{CDH1} complex (Huang et al., 2011). Similarly, levels of USP33 oscillate during the cell cycle, which has implications for USP33-mediated stabilisation of CP110, a centriolar protein that controls cell cycle-dependent centrosome duplication (Li et al., 2013). Transcription of A20 and cylindromatosis (CYLD), modulators of NFκB signalling, is also tightly regulated and induced in response to NFκB activation in a feedback loop to inhibit the pathway (Pahl, 1999; Sun, 2010). Moreover, several DUBs are ubiquitylated and some, such as USP4 (Zhang et al., 2012), can autodeubiquitylate and hence counteract their own ubiquitin-mediated degradation. Therefore, transcriptional and post-translational mechanisms regulate the abundance of DUBs and thus their functions. This regulation can also be cell type-specific, enabling fine tuning of ubiquitin signalling.

Interacting partners of DUBs

Interacting partners are key regulators of the biological functions of DUBs that can affect their catalytic activity and facilitate substrate recognition. Indeed, in some cases, a DUB is essentially inactive unless it is incorporated into a protein complex. This is particularly evident in the case of large macromolecular assemblies that contain DUBs as functional subunits. For example, all three proteasome-associated DUBs, the metalloprotease Rpn11 and the two cysteine proteases USP14 and UCH37, are significantly activated upon incorporation into the 19S regulatory particle (Borodovsky et al., 2001; Qiu et al., 2006; Worden et al.,

2014; Yao et al., 2006). Recruitment of these DUBs not only enhances their catalytic activity but also places them within the context of the proteasome where they can influence different stages of protein degradation. Hence, USP14 and UCH37 can potentially modulate the ubiquitylation status of proteins prior to their commitment for degradation, whilst Rpn11 acts at later stages (Worden et al., 2014). Interestingly, UCH37 is also part of another complex, the chromatin-remodelling INO80 complex, in which its activity is inhibited (Yao et al., 2008). Elegant structural studies revealed that equivalent DEUBiquitylase ADaptor (DEUBAD) domains present in both Rpn13 and INO80G induce distinct structural rearrangements in UCH37 that result in its activation at the proteasome and inhibition at the INO80 complex, respectively (Sahtoe et al., 2015; VanderLinden et al., 2015).

Akin to the recruitment of UCH37 into two distinct complexes, the proteasome and the INO80 complex, BRCC36 (a member of the JAMM DUB family) is incorporated into two alternative complexes, the nuclear Abraxas complex (ARISC) and cytosolic BRCC36 isopeptidase complex (BRISC). Both ARISC and BRISC complexes share the core subunits, BRCC45 and MERIT40, but in addition they also contain complex-specific components: Abro1 (also known as KIAA0157) for the BRISC complex and Abraxas for the ARISC complex (Feng et al., 2010). On its own, BRCC36 is largely inactive, whereas, as part of the cytosolic BRISC complex, BRCC36 is activated by its subunit Abro1 (Feng et al., 2010; Zeqiraj et al., 2015). In contrast, activation of BRCC36 within the nuclear ARISC requires concerted action of its core components, as well as the Abraxas and RAP80 subunits (Feng et al., 2010). Importantly, incorporation of BRCC36 into these two distinct complexes defines its intracellular localisation and biological function. As a result, ARISC-incorporated BRCC36 functions in DNA damage repair (Shao et al., 2009), whereas BRISC-recruited BRCC36 stabilises type I interferon receptor by preventing its endocytosis and lysosomal degradation (Zheng et al., 2013), and affects mitotic spindle assembly by deubiquitylating nuclear mitotic apparatus protein 1 (NuMA) (Yan et al., 2015).

The Spt-Ada-Gcn5-Acetyl transferase (SAGA) complex is another multisubunit assembly that ensures the correct localisation and substrate recognition by a DUB, in this case USP22. The SAGA complex acts as a transcriptional coactivator by catalysing acetylation and deubiquitylation of histone H2B. This latter activity is mediated by the SAGA deubiquitylating module (mDUB), which in mammals, is composed of the subunits USP22, ATXN7L3, ENY2 and ATXN7 (yeast Ubp8, Sgf11, Sus1 and Sgf73, respectively) (Koutelou et al., 2010). Importantly, whilst USP22 (Ubp8) is the only DUB, the remaining three

components within the mDUB are also critically required for the deubiquitylating activity (Lee et al., 2005). The crystal structures of yeast mDUB provided a rationale for this by revealing that all subunits form extensive contacts with each other in a manner that could stabilise and activate Ubp8 (Kohler et al., 2010; Samara et al., 2010). Interestingly, ATXN7L3 and ENY2 of mammalian mDUB also bind to and activate two additional DUBs, USP27X and USP51, although not as part of the SAGA complex (Atanassov et al., 2016). This suggests that these two non-catalytic subunits of mDUB could act as master regulators of histone H2B deubiquitylation by multiple DUBs.

Additional examples of the widespread modulation of DUB functions by interacting partners include the regulation of the catalytic activities of USP1, USP12 and USP46 by USP1-associated factor 1(UAF1) and WD repeat-containing proteins (Cohn et al., 2009; Kee et al., 2010). Similarly, interaction of USP10 with the Ras-GTPase activating protein G3BP affects the catalytic activity of USP10 and the G3BP-USP10 complex regulates the formation of stress granules in response to phosphorylation of eukaryotic initiation factor 2α or inhibition of eukaryotic initiation factor 4A (Kedersha et al., 2016; Soncini et al., 2001). Furthermore, spermatogenesis-associated protein 2 (SPATA2) acts as a scaffold protein that links CYLD to HOIL-1L-interacting protein (HOIP), an E3 ligase component of the linear ubiquitin chain assembly complex (LUBAC), and so controls the outcome of tumour necrosis factor receptor 1 (TNFR1)-mediated signalling (Elliott et al., 2016; Kupka et al., 2016; Schlicher et al., 2016; Wagner et al., 2016). In a similar manner, interactions of USP8 and associated-molecule-with-the-SH3-domain-of-signal-transducing-adapter-molecule-1 (AMSH) with signal-transducing-adapter-molecule-1 (STAM) and endosomal-sorting-complex-required-for-transport-III (ESCRT-III) components ensure the endosomal localisation of these DUBs and thereby support their function in endocytosis (Clague and Urbe, 2017; Millard and Wood, 2006). The examples of DUB interactions listed above highlight the importance of binding partners in regulating DUB biology and show how the same DUB can perform different functions depending on its interacting proteins. We anticipate that such regulation is widely prevalent, and it will be important to study DUBs within the context of such complexes and their constituent proteins.

Regulating DUB function by antagonising ubiquitylation

In addition to interacting with scaffold proteins, DUBs are also often found in complexes with other components of the ubiquitin system such as E3 ubiquitin ligases (Sowa et al., 2009). The presence of such complexes can reflect several scenarios. For example, DUBs can reverse autoubiquitylation of E3 ligases and prevent their degradation. Moreover, DUBs are also targets of E3 ligase-mediated ubiquitylation (Huang et al., 2011; Mashtalir et al., 2014; Scaglione et al., 2011; Seki et al., 2013; Todi et al., 2009; Wijnhoven et al., 2015). Importantly, in recent years it has become clear that DUBs and E3 ligases can co-operate to dictate the outcome of protein ubiquitylation. In such a scenario, a DUB limits or modulates the linkage type of polyubiquitin generated by an E3 ubiquitin ligase (Fig. 2). For example, the monoubiquitylated form of the E3 ubiquitin ligase carboxy-terminus-of-Hsp70-interacting-protein (CHIP) recruits the DUB ataxin-3, which limits the elongation of polyubiquitin on CHIP substrates (Scaglione et al., 2011). Importantly, such an editing of polyubiquitylation appears to be important for the degradation of CHIP substrates, as expression of catalytically inactive ataxin-3 or mutation of the ubiquitylation site on CHIP that serves to recruit ataxin-3 both perturb the degradation of CHIP substrates (Scaglione et al., 2011). Ataxin-3 also deubiquitylates CHIP upon completion of substrate ubiquitylation, therefore contributing to the termination of the ubiquitylation cycle.

In a similar manner, the E3 ligase LUBAC interacts with two DUBs capable of cleaving linear polyubiquitin, OTULIN and CYLD (Takiuchi et al., 2014). This interaction occurs through the N-terminal PUB domain of the HOIP subunit of LUBAC and is critical in controlling the *in vitro* generation and *in vivo* levels of linear ubiquitin chains (Elliott et al., 2014; Schaeffer et al., 2014). Importantly, interaction of HOIP with these two DUBs plays a key role in inhibiting the extent of NFκB activation following TNFα treatment. By being associated with E3 ubiquitin ligases, DUBs can negatively regulate E3 ligase activity, and thus activation of the E3 would require dissociation or inhibition of the DUB (Fig. 2). In an alternative model, DUBs cleave certain linkage types to edit the ubiquitin signal that has been assembled by the E3 ligase, which results in a different linkage type or monoubiquitylation of the substrate (Fig. 2). In both scenarios, co-operation between a DUB and an E3 ubiquitin ligase can orchestrate the strength and functional outcome of ubiquitin signal.

Otubain-1 (OTUB1) employs yet another mechanism to antagonise protein ubiquitylation. Although OTUB1 is a DUB with an enzymatic activity that is specific for Lys48-linked chains, it can also suppress protein ubiquitylation in a manner that is independent of its catalytic activity by binding to ubiquitin-charged E2 enzymes such as UBE2N (Nakada et al.,

2010). As revealed by structural studies, the binding of OTUB1 to UBE2N inhibits ubiquitylation by blocking the formation of an isopeptide bond between donor and acceptor ubiquitin, hindering access to the E3 enzyme and preventing interaction between donor ubiquitin and the E2 enzyme (Juang et al., 2012; Wiener et al., 2012). This non-catalytic function of OTUB1 plays an important role in suppressing Lys63-linked ubiquitylation of chromatin at DNA double-strand breaks in response to, for example, ionising radiation (Nakada et al., 2010). Interestingly, OTUB1 also interacts with other E2 enzymes that can assemble ubiquitin chains distinct from Lys63-linked ones suggesting that OTUB1-mediated inhibition of ubiquitylation might have a wide-spread function (Nakada et al., 2010). Expanding on the theme of catalytic-independent modulation of protein ubiquitylation, there are many DUBs that lack key catalytic residues and are predicted to be inactive. We speculate that due to the presence of an otherwise intact DUB domain, these pseudoDUBs might compete with catalytically active DUBs for substrate or could function as ubiquitin-binding domains. Analogous to UBE2N-UBE2V1, the E2-pseudoE2 pair that work together to assemble Lys63-linked polyubiquitin, one could envisage pseudoDUBs enhancing or inhibiting the activity of DUBs.

In summary, interactions between DUBs and their partners regulate many aspects of DUB biology, such as their catalytic activities and substrate recognition. Interactions with other proteins also constitute one of several mechanisms that define the intracellular localisation of DUBs – another aspect instrumental for DUBs to fulfil their biological roles. Given the profound effect that interacting partners exert on DUB functions it will be crucial to understand their identities under various conditions and in different types of cells or tissues.

Localisation-dependent DUB functions

The subcellular localisation of DUBs (Fig. 3) has several important consequences for their functions. It defines, for example, access of a DUB to a specific substrate and, therefore, roles within a particular physiological pathway. This is especially evident in case of the transmembrane DUBs USP19 and USP30 whose intracellular localisation is highly restricted. Endoplasmic reticulum (ER) localisation of USP19 is crucial for its function in the ER-associated degradation pathway (ERAD) ((Hassink et al., 2009) but see also (Lee et al., 2014) where USP19 involvement in ERAD has been questioned), secretion of misfolded proteins upon proteasome dysfunction (Lee et al., 2016) and ER exit of membrane proteins that fold

inefficiently (Perrody et al., 2016). Similarly, USP30, which is localised to the mitochondrial outer membrane, regulates mitochondrial morphology (Nakamura and Hirose, 2008) and has been implicated in Parkin-mediated mitophagy (Bingol et al., 2014; Liang et al., 2015). In the same manner, nucleolar localisation of USP36 allows it to specifically interact with and deubiquitylate a nucleolar pool of c-Myc (Sun et al., 2015), whereas endosome-localised AMSH and USP8 regulate the lysosomal degradation and recycling of receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) (Clague and Urbe, 2017; Millard and Wood, 2006).

In principle, the repertoire of DUB substrates, and hence the biological functions of DUBs, can be expanded by ensuring that a single DUB is present at alternative locations within the cell. Indeed, a number of DUBs were shown to simultaneously localise to distinct intracellular structures. A computational analysis identified potential nuclear export signals (NESs) in many DUBs that would allow for their shuttling between the nucleus and cytosol (Garcia-Santisteban et al., 2012), a prediction that has been experimentally validated for USP21(Garcia-Santisteban et al., 2012). Indeed, the presence of such a NES might account for the dual role of USP21 in regulating transcription via histone H2A deubiquitylation in the nucleus (Nakagawa et al., 2008) and in primary cilia formation and cilia-related Hedgehog signalling when it is localised to the centrosome (Heride et al., 2016; Urbe et al., 2012). In a similar manner, USP12 deubiquitylates histones H2A and H2B (Joo et al., 2011), but also translocates from the nucleus following T cell receptor (TCR) stimulation to deubiquitylate and stabilise the TCR adaptor proteins linker-for-activation-of-T-cells-family-member-1 (LAT) and T-cell-receptor-associated-transmembrane-adapter-1 (Trat1) at the cell surface (Jahan et al., 2016).

Alternative localisation is also intrinsically linked to the function of CYLD, which is recruited to the necrosome complex where it deubiquitylates receptor-interacting-serine/threonine-protein-kinase-1 (RIP1) and contributes to necroptosis progression (Liu et al., 2016). At the same time, CYLD can also interact with several centrosomal proteins, such as CEP192 (Gomez-Ferreria et al., 2012), CAP350 (Eguether et al., 2014) and CEP70 (Yang et al., 2014b), and this centrosomal pool of CYLD has been shown to regulate primary cilia formation and mitotic spindle assembly and/or orientation (Eguether et al., 2014; Gomez-Ferreria et al., 2012; Yang et al., 2014a; Yang et al., 2014b). Similarly, USP15 deubiquitylates histone H2B in the nucleus (Long et al., 2014), prevents degradation of the transcriptional repressor RE1-silencing-transcription-factor (REST) at the ribosome

(Faronato et al., 2013) as well as opposes RNF26-mediated ubiquitylation of p62 at the ER, thus regulating the mobility of endosomes (Jongsma et al., 2016). These examples highlight how DUBs can exert different functions just by being at different places, a theme that is being increasingly explored.

Post-translational modifications

The function of many, if not most, proteins is regulated by their post-translational modifications (PTMs). This is also true for DUBs which are targets of many PTMs such as phosphorylation, ubiquitylation, SUMOylation, lipidation and oxidation, all of which can affect the biology of DUBs and are discussed here.

Phosphorylation

Phosphorylation is a key post-translational modification that affects the function of a number of DUBs, highlighting the crosstalk between enzymes that regulate different PTMs, in this case phosphorylation and ubiquitylation. Phosphorylation has been observed to either activate, inhibit or modulate the catalytic activity of DUBs (Fig. 4A). Deubiquitinating enzyme A (DUBA, also known as OTUD5) is a negative regulator of immune responses (Kayagaki et al., 2007). For a long time, it has been puzzling as to why bacterially expressed recombinant DUBA was not an active enzyme (Huang et al., 2012). An answer to this came from the demonstratation that casein kinase 2 (CK2)-mediated phosphorylation at Ser177 within the catalytic domain activates DUBA (Huang et al., 2012), representing the first example for how phosphorylation could activate a DUB.

USP14 can also be activated by phosphorylation in a manner that is distinct from its proteasome-dependent activation (Xu et al., 2015) (see also above). When phosphorylated by RAC- serine/threonine-protein-kinase (AKT) at Ser432, USP14 is activated independently of the proteasome and is then able to cleave polyubiquitin that is linked via Lys48 to stabilise proteasomal substrates or Lys63 to control non-degradative outcomes of ubiquitylation. For example, by removing Lys63-linked polyubiquitin from Beclin-1, phosphorylated USP14 decreases the association of Beclin-1 with Atg14L and UV-radiation-resistance-associated-gene-protein (UVRAG), and so downregulates autophagy (Xu et al., 2016).

USP10, USP37 and USP9X are further examples of DUBs that are activated by phosphorylation. Phosphorylation at Ser76 by AMP-activated-protein-kinase (AMPK) activates USP10, which can then remove Lys63-linked polyubiquitin from the activation loop

of AMPK (Deng et al., 2016). This in turn enables liver-kinase-B1 (LKB1)-mediated phosphorylation of AMPK at Thr172, leading to further AMPK activation. Hence, AMPK-dependent phosphorylation of USP10 establishes a positive feedback loop for enhancing AMPK activity during conditions of energy stress. Consistent with such a scenario USP10 depletion in mouse liver leads to metabolic defects such as an increase in triglyceride and cholesterol content (Deng et al., 2016). By contrast, USP37 is activated in a cell cycle-specific manner by CDK2-mediated phosphorylation at Ser628 (Huang et al., 2011). This regulated activation is fundamental for its ability to antagonise APC^{CDH1}-mediated degradation of cyclin A, thus controlling cell cycle progression into S phase (Huang et al., 2011). In case of USP9X its phosphorylation at Ser1600 occurs in response to TCR activation and enhances its catalytic activity (Naik and Dixit, 2016). This in turn facilitates USP9X interactions with components of the TCR signalling cascade and ultimately leads to deubiquitylation of ZAP70 preventing its sequestration in early endosomes (Naik and Dixit, 2016).

Phosphorylation can also inhibit the catalytic activity of DUBs as shown for CYLD upon its phosphorylation at Ser418 by inhibitor-of-nuclear-factor-kappa-B-kinase-subunit-epsilon (IKKε) (Hutti et al., 2009). In this case, phosphorylated CYLD is less efficient at removing polyubiquitin from its substrates, for example TRAF2, following TNFα stimulation. Importantly, phosphorylation-deficient CYLD significantly limits the size of tumours resulting from the introduction of IKKε-transformed cells into immunodeficient mice (Hutti et al., 2009). This in turn suggests that CYLD phosphorylation is required to induce oncogenic transformation mediated by IKKε (Hutti et al., 2009).

An interesting example of phosphorylation-mediated regulation of DUB function is illustrated by A20, an OTU-domain containing DUB. A20 is unique amongst DUBs as it combines DUB and E3 ubiquitin ligase activities within a single polypeptide chain (Wertz et al., 2004). Hence, A20 removes Lys63-linked polyubiquitin from RIP1 and, by assembling Lys48-linked chains, mediates its proteasomal degradation. This in turn shifts the outcome of RIP1 ubiquitylation from a prosurvival to a proapoptotic signal (Wertz et al., 2004). This activity is controlled by phosphorylation of A20 on Ser381 within the Zinc Finger region by IKKβ, which enhances the hydrolysis of Lys63-linked polyubiquitin and activates the E3 ubiquitin ligase activity (Wertz et al., 2015).

In addition to directly affecting the catalytic activity of DUBs, phosphorylation can also indirectly regulate DUB function, for example, by changing their subcellular localisation. For instance, phosphorylation of USP4 at Ser445 that is mediated by AKT triggers USP4 translocation from the nucleus to the cytosol and intracellular membranes (Zhang et al., 2012). This enables USP4-catalysed deubiquitylation of transforming-growth-factor-beta (TGFβ) type I receptor, which in turn extends its half-life at the plasma membrane and induces TGFβ signalling. This function of USP4 has been shown to stimulate TGFβ-induced breast cancer cell invasion and metastasis (Zhang et al., 2012). Similarly, when phosphorylated at Ser16 by CK2, OTUB1 accumulates in the nucleus, and its phosphorylation status impacts on the formation of 53BP1-positive foci following ionising radiation-induced DNA damage (Herhaus et al., 2015). Phosphorylation is therefore emerging as an important regulator of DUB function, as it not only regulates the activity of DUBs, but also their interactions and subcellular localisation.

Ubiquitylation and SUMOylation

Modification of DUBs with ubiquitin and ubiquitin-like molecules constitutes another mode of regulating their activities (Fig. 4A). For example, monoubiquitylation of ataxin-3, a DUB implicated in Spinocerebellar Ataxia Type 3, enhances its catalytic activity (Todi et al., 2009). Ubiquitylation of JosD1, another MJD family DUB, in addition to activating it also leads to its relocalisation from the cytoskeletal to the membrane fraction as assessed by subcellular fractionation (Seki et al., 2013). In an analogous manner, UBE2O-mediated ubiquitylation of lysine residues located in the proximity of the nuclear localisation sequence of BRCA1-associated protein 1 (BAP1) leads to an accumulation of ubiquitylated BAP1 in the cytosol (Mashtalir et al., 2014). Interestingly, BAP1 can also mediate its own deubiquitylation, and BAP1 mutations that result in increased levels of its ubiquitylated form have been linked to cancer (Mashtalir et al., 2014). A similar autodeubiquitylation event has been described for USP4, a DUB implicated in the DNA damage repair pathway (Wijnhoven et al., 2015). Interestingly, USP4 is modified by unconventional cysteine ubiquitylation, which blocks its interaction with the key DNA damage repair proteins CtBP-interacting protein (CtIP) and RAD50 (Wijnhoven et al., 2015). The same report also suggests that a related DUB, USP15, is similarly ubiquitylated on a cysteine residue and this modification affects its interaction with the TGFβ signalling pathway components SMAD2 and SMAD3

(Wijnhoven et al., 2015). Furthermore, USP15 can also promote its own autodeubiquitylation (Wijnhoven et al., 2015).

SUMOylation is closely related to ubiquitylation and has also been shown to regulate the activities of DUBs. USP25 is modified by SUMO preferentially at lysines 99 and 141, which are located within the first ubiquitin interacting motif (UIM) and adjacent to the second UIM, respectively (Meulmeester et al., 2008). This modification inhibits polyubiquitin hydrolysis by USP25, most likely by blocking the binding of ubiquitin to its UIM. Similarly, SUMOylation of CYLD inhibits CYLD-mediated deubiquitylation of components of the NFκB signalling pathway (Kobayashi et al., 2015).

Other modifications

Several other PTMs have been implicated in regulating the biology of DUBs. For example, farnesylation of UCH-L1 promotes its association with intracellular membranes and stimulates the accumulation of α-synuclein, which has been linked to Parkinson's disease, in neuroblastoma cells (Liu et al., 2009). The DUBs USP32, MINDY-1 and MINDY-2 are also predicted to be modified with lipids, which might account for the association of USP32 with intracellular membranes (Abdul Rehman et al., 2016; Akhavantabasi et al., 2010). Finally, reversible oxidation of the catalytic cysteine of DUBs that inhibits their activity has also been reported (Cotto-Rios et al., 2012; Kulathu et al., 2013; Lee et al., 2013); this could provide an attractive model for how ubiquitin signals can be amplified when DUB activity is reversibly inhibited in a spatio-temporal manner in response to production of reactive oxygen species.

Expanding DUB functionality with DUB isoforms

A median of five isoform transcripts has been reported for human genes, although this number can vary significantly (Floor and Doudna, 2016). Such alternative splicing and/or the use of alternative transcription initiation sites constitute an attractive mode of how the biological functions of DUBs can be further expanded. This is particularly important in light of a recent study suggesting that alternative protein isoforms behave more like unrelated proteins, rather than slight variations of the same polypeptide (Yang et al., 2016). Importantly, depending on the tissue analysed, alternative isoforms might be actually more abundant than the so-called canonical ones (Yang et al., 2016).

The following examples highlight why it is important to consider isoforms when studying DUBs. As with many other proteins, the functional expansion of DUBs by their various isoforms remains largely unexplored. One notable exception is USP19, for which two isoforms have been described that differ in their C-terminal region. As a consequence, one isoform contains a membrane-spanning region and is embedded within the ER membrane, whereas the second isoform is cytoplasmic. Importantly, the cytosolic isoform cannot substitute for the ER-localised one in processes, such as ER-associated degradation or secretion of misfolded proteins (Hassink et al., 2009; Lee et al., 2016). A similar isoform-specific localisation was observed for USP33, for which at least three different isoforms have been reported (Thorne et al., 2011). Although all of these associate with the ER, isoform 3 accumulates at the Golgi apparatus due to a deletion of eight amino acids that act as a "Golgi exclusion signal" (Thorne et al., 2011). Reminiscent of the isoform-specific localisation of USP33, USP35 has also been suggested to exist as two isoforms that localise either to the cytosol or mitochondria (Wang et al., 2015). The latter isoform contains a predicted mitochondria-targeting sequence and has been suggested to have a role in mitophagy (Wang et al., 2015). However, the existence of the transcript or protein that corresponds to the putative USP35 mitochondrial isoform has not been confirmed experimentally and its potential function thus remains speculative at this point.

Finally, two isoforms of OTUB1 that differ in their N-terminal regions due to the use of alternative translation initiation sites have also been reported. Catalytically active OTUB1 is ubiquitously expressed, whereas the second catalytically-inactive isoform, called ARF-1, is predominantly produced in tissues of the immune system, such as tonsils and lymph nodes (Soares et al., 2004). Both isoforms interact with the E3 ubiquitin ligase gene-related-to-anergy-in-lymphocytes-protein (GRAIL), but, surprisingly, OTUB1 destabilises GRAIL, whereas the catalytically inactive isoform ARF-1 increases GRAIL levels. This might be due to differential, isoform-specific, regulation of USP8 activity, another DUB proposed to directly deubiquitylate GRAIL. Consistent with their differing effects on GRAIL, the two OTUB1 isoforms have opposing roles in anergy and cytokine production following T cell activation (Soares et al., 2004).

Interestingly, a recent high-throughput study indicates that mRNAs corresponding to multiple DUB isoforms associate with translating ribosomes in HEK293 cells, suggesting that they are indeed produced (Floor and Doudna, 2016). However, for most of these transcripts the 5'- or 3'-coding sequences are not annotated, making it difficult to unambiguously determine the

amino acid sequence of these isoforms. Nonetheless, the generation of multiple isoforms by alternative splicing could be an important mechanism for not only expanding the repertoire of available DUBs, but also for ensuring the dynamic regulation of ubiquitylation in a cell-type- or tissue-specific and stimulus-dependent manner.

Regulation of DUB function by proteolytic processing

Alternative splicing often results in the generation of isoforms that correspond to N- or C-terminal truncations of the "canonical" isoform. A similar effect could in principle be achieved post-translationally by proteolytic processing of DUBs, and indeed this mode of regulation has recently emerged as yet another mechanism of regulating DUB function (Fig. 4B). For example, USP7 is cleaved by caspase-3 during dexamethasone-triggered apoptosis in foetal thymic organ culture (Vugmeyster et al., 2002). Such proteolysis of USP7 generates a truncated protein that is still active as evidenced by its reactivity with a DUB-selective probe that modifies the catalytic cysteine of an active DUB. The role of this cleavage is unknown, but it could potentially alter the interactions of USP7 with other proteins during apoptosis. The DUB USP8 also undergoes proteolytic processing and several mutations of USP8 that cluster in the vicinity of its binding site for the 14-3-3 proteins were identified in Cushing's disease tumours (Ma et al., 2015; Reincke et al., 2015). These mutations disrupt the interaction of USP8 with 14-3-3 proteins, previously reported to inhibit USP8 catalytic activity (Mizuno et al., 2007), and make USP8 susceptible to the action of an unknown protease, which cleaves USP8 between Lys714 and Arg715 (Reincke et al., 2015). This, in turn, leads to release of a C-terminal USP8 fragment (tUSP8) that has increased catalytic activity (Reincke et al., 2015). The tUSP8 variant can still interact with EGFR and is very efficient at deubiquitylating EGFR. Disease mutants result in truncated USP8 that now promotes EGFR recycling to the plasma membrane, instead of downregulating the receptor. Interestingly, USP8 is also cleaved in a caspase-dependent manner in T cells following TCR activation (Dufner et al., 2015).

Proteolytic processing of DUBs is also employed to block their biological activities. Caspase-8-mediated cleavage of CYLD at Asp215 serves to inactivate CYLD and confers a prosurvival signal during necroptosis (O'Donnell et al., 2011). Interestingly, whilst the caspase-8-mediated cleavage occurs outside the catalytic domain of CYLD, the released C-terminal fragment containing its USP domain is unstable and is degraded by the proteasome.

This CYLD proteolysis is important to inhibit necroptosis in macrophages stimulated with LPS (Legarda et al., 2016).

A20 is another DUB that is inactivated by proteolytic cleavage (Coornaert et al., 2008). Processing of A20 by a paracaspase mucosa-associated-lymphoid-tissue-lymphoma-translocation-protein-1 (MALT1) at Arg439 generates an intact OTU domain that is unstable (Coornaert et al., 2008). A20 cleavage occurs in response to TCR stimulation and impairs its role in inhibiting NF-κB activation (Coornaert et al., 2008).

An interesting mechanism for inactivating a DUB by proteolytic cleavage was shown for USP1, a DUB that deubiquitylates monoubiquitylated PCNA in the translesion synthesis pathway, and monoubiquitylated Fanconi Anemia group I protein (FANCI) and Fanconi Anemia group D2 protein (FANCD2) of the Fanconi Anemia interstrand crosslink repair pathway (Nijman et al., 2005a; Sims et al., 2007; Smogorzewska et al., 2007). Upon ultraviolet irradiation, USP1 undergoes autocleavage, thus resulting in the accumulation of monoubiquitylated PCNA, an event important for DNA damage repair (Huang et al., 2006). Strikingly, this autocleavage event occurs immediately after a Gly-Gly repeat, a motif also found in linear ubiquitin fusions such as ubiquitin precursors (Baker and Board, 1991; Lund et al., 1985; Redman and Rechsteiner, 1989; Wiborg et al., 1985). Because numerous other DUBs also contain Gly-Gly motifs, autocleavage to modulate DUB activity could be more widespread than currently appreciated. Importantly, as is the case for the autocleaved USP1 (Piatkov et al., 2012), proteolytically processed DUBs might expose destabilising residues at their newly generated N-terminus, which would lead to their degradation via the N-end rule pathway.

Conclusions and perspectives

DUBs are key regulators of a plethora of intracellular processes and as such have been implicated in the pathogenesis of a number of human disorders (Heideker and Wertz, 2015). Accordingly, DUBs have been a topic of intense research, and recent years have seen an explosion in the number of reports addressing their mechanisms of action and biological roles. However, we are still very far from having a complete picture of DUB biology. One of the main unanswered questions is how do the small number of approximately 100 enzymes modulate a multitude of ubiquitylation events that occur in eukaryotic cells? We postulate that an expansion of DUB function via the many mechanisms discussed here will be crucial

for the regulation of ubiquitylation. Clearly, further work is needed to fully understand the extent to which such functional expansion is employed. For example, analysis of transcription and translation of DUB isoforms in various organs and under a range of experimental conditions will be invaluable for our understanding of the total number of different DUB polypeptides that are produced and that could have distinct functional capabilities. Moreover, exploitation of techniques to identify transient and weak affinity protein-protein interactions will be instrumental in defining the atlas of DUB regulators and interactors. This will help to reveal the full set of biological pathways in which DUBs participate, their regulation within these pathways, and thus a full appreciation of DUB function in eukaryotic cell biology. We also anticipate that the outcome of DUB-focused research will contribute to deciphering the molecular basis of the pathogenesis of human disorders and thus lead to novel or improved therapeutic strategies. We hope that the paradigms presented in this Commentary of how diversification and regulation of DUB function is achieved will guide future research.

FIGURE LEGENDS

Fig. 1. Overview of protein ubiquitylation.

Ubiquitin is activated and transferred to a substrate protein in a cascade reaction catalysed by E1 activating, E2 conjugating and E3 ligating enzymes. This can lead to the formation of distinct types of modifications, all of which can potentially be reversed by deubiquitylating enzymes (DUBs). Numbers in brackets indicate the number of members for each family of enzymes.

Fig. 2. Mechanisms of DUB-mediated modulation of protein ubiquitylation.

(A) DUBs can cleave polyubiquitin either from the distal end (exoDUBs) or within the chain (endoDUBs). **(B)** DUBs act in concert with E3 ubiquitin ligases to modulate protein ubiquitylation. For example, partial deubiquitylation can leave a ubiquitin moiety attached to a substrate, which is then extended by an E3 ligase to form polyubiquitin of a distinct linkage type. **(C-D)** Alternatively, a DUB can remove a certain linkage type (shown in brown) or limit the length of the polyubiquitin chain ligated to a substrate. (E) DUBs can also constitutively counteract some E3 ligases whose activation requires DUB dissociation and/or inactivation.

Fig. 3. Intracellular localisation of DUBs.

DUBs have been reported to function in almost every intracellular compartment and have localisation-specific roles. Importantly, DUB functionality can be expanded by ensuring that a single DUB localises to distinct organelles as shown, for example, for USP15 and USP21. Such alternative localisation can be regulated by DUB interacting partners, post-translational modifications and alternative splicing, and the predominant steady-state localisation presumably depends on the experimental conditions used. The figure was generated based on both the localisation studies where available and the reported activities of DUBs at the indicated locations.

Fig. 4. Additional modes of DUB regulation

(A) DUB function can be regulated by post-translational modifications (PTMs) that affect either DUB localisation or their catalytic activity. For example, phosphorylation of USP4 by AKT re-localises it from the nucleus to the cytosol. In contrast, CK2-mediated phosphorylation of OTUB1 triggers its translocation from the cytosol to the nucleus. DUB activities can also be modulated by PTMs such as phosphorylation, ubiquitylation and SUMOylation. The enzymes catalysing PTMs of selected DUBs are named above the arrows. **(B)** DUBs can also be proteolytically processed. This can lead to their degradation as exemplified by CYLD, which is cleaved by caspase-8 following the TNF receptor 1 (TNFR1) ligation (top). Alternatively, DUB proteolysis can result in a functional protein (illustrated below). The truncated form of USP8 (tUSP8) is present in some Cushing's disease tumours and has increased catalytic activity. Like USP8, tUSP8 interacts with EGFR, and, owing to more efficient deubiquitylation, promotes EGFR recycling to the plasma membrane, resulting in sustained proliferative signalling.

Table 1. DUBs are divided into several families.

DUBs can be divided into distinct families that include ubiquitin-specific proteases (USPs), ubiquitin C-terminal hydrolases (UCHs), ovarian tumour proteases (OTUs), Machado-Joseph disease proteases (MJDs), MIU-containing novel DUB family (MINDY) proteases and the JAB1/MPN/Mov34 (JAMM) domain proteases. DUBs are cysteine proteases with the exception of the JAMM family that is composed of metalloproteases. Numbers of active and inactive members of each family of DUBs are given.

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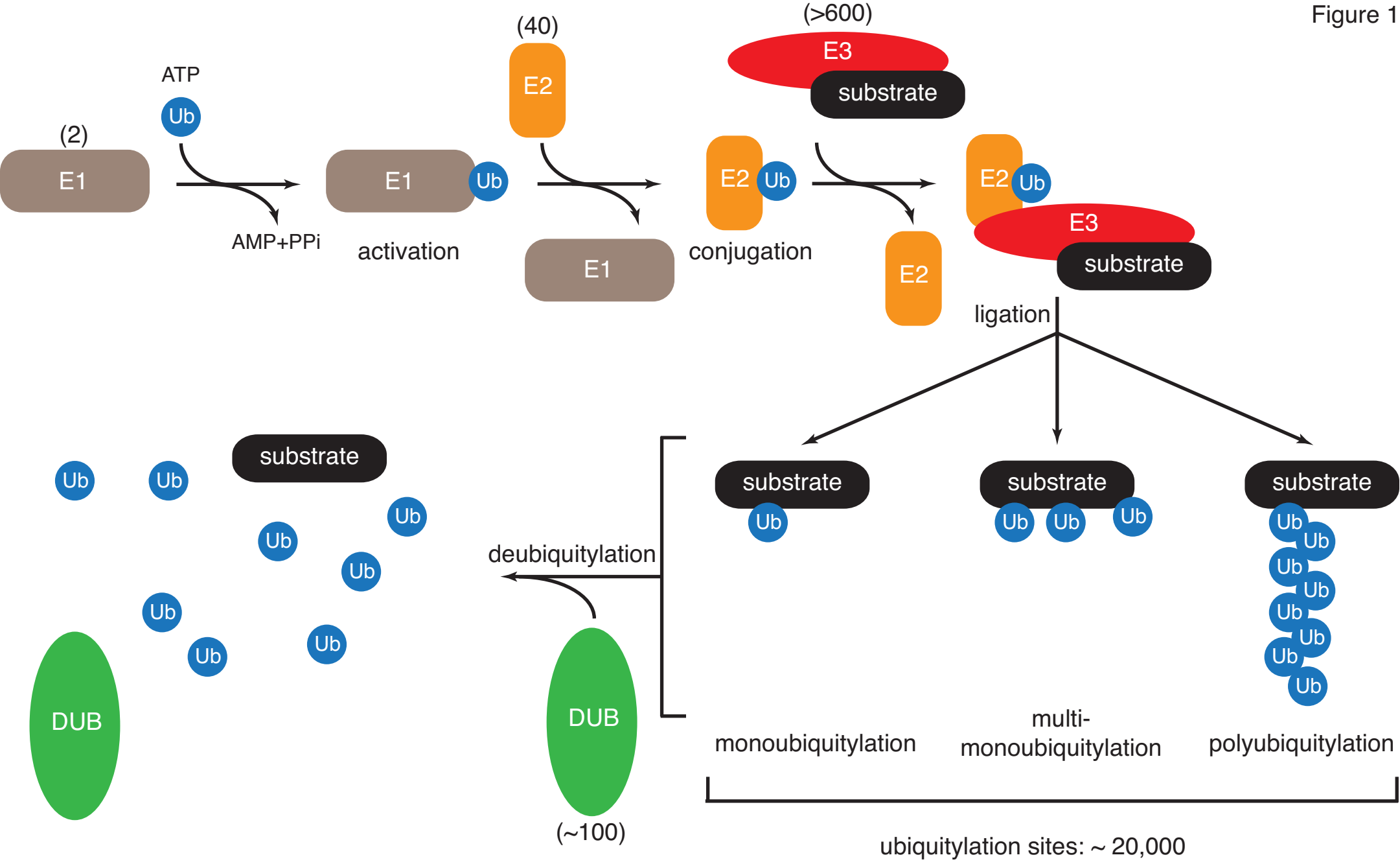


Figure 1
(2)
E1
ATP
Ub
AMP+PPi
E1
Ub
activation
(40)
E2
E1
(>600)
E3
substrate
E2
Ub
conjugation
E2
E2
Ub
E3
substrate
ligation
substrate
Ub
Ub
Ub
Ub
Ub
Ub
Ub
DUB
deubiquitylation
DUB
(~100)
substrate
Ub
substrate
Ub
Ub
Ub
substrate
Ub
Ub
Ub
Ub
Ub
Ub
Ub
Ub
Ub
monoubiquitylation
multi-
monoubiquitylation
polyubiquitylation
ubiquitylation sites: ~ 20,000

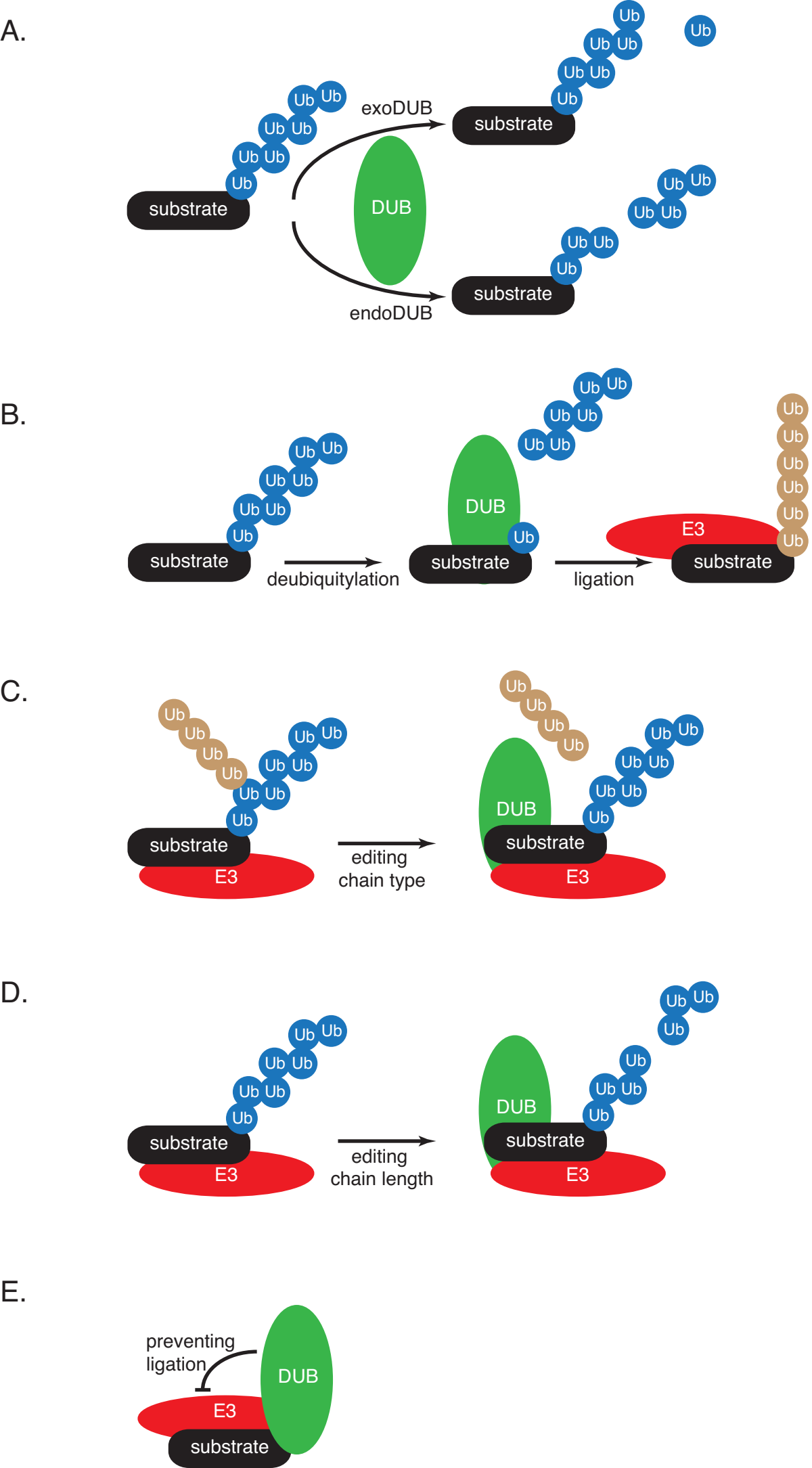
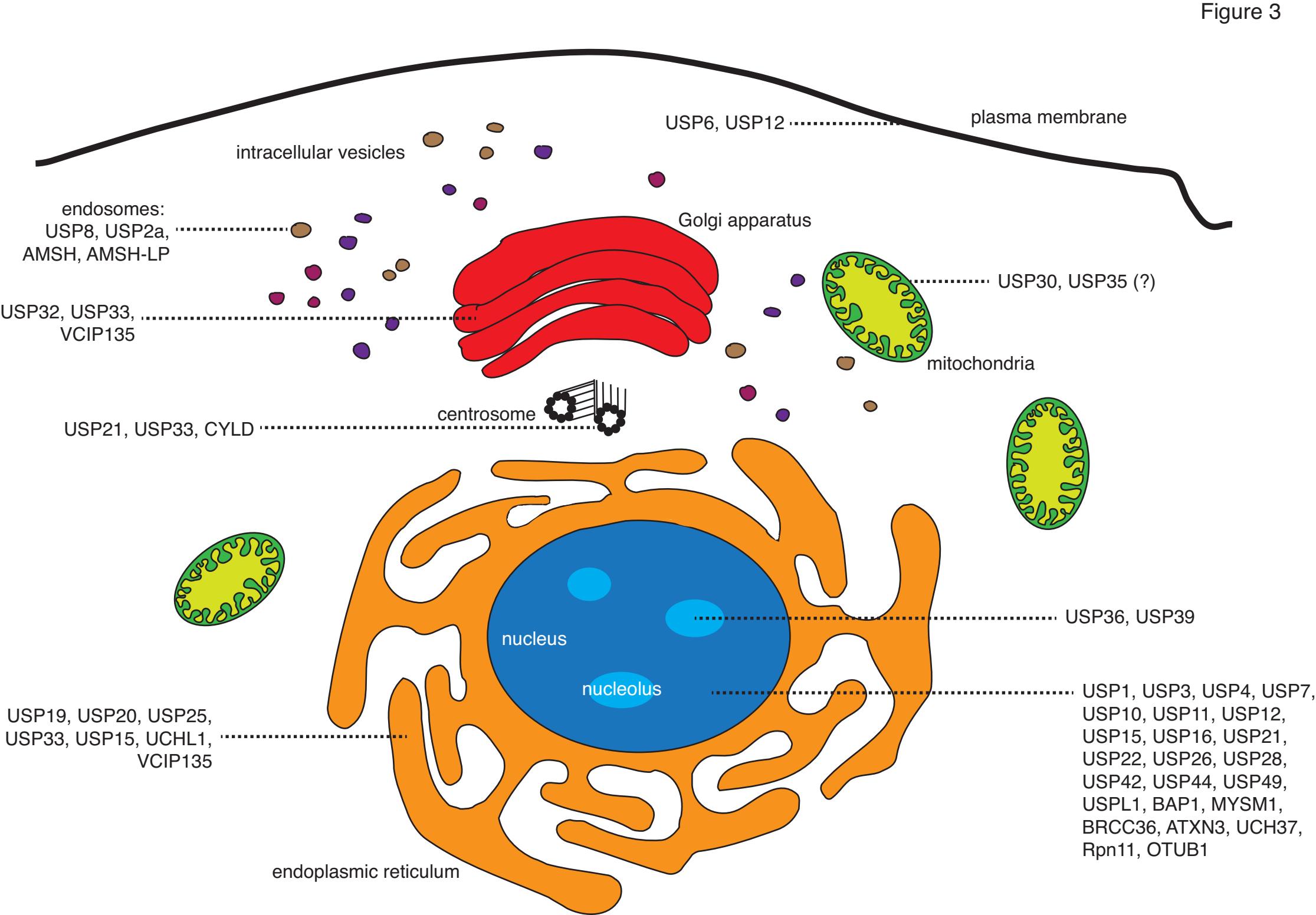

A.
Ub
substrate
exoDUB
DUB
endoDUB
B.
deubiquitylation
ligation
E3
C.
editing
chain type
D.
editing
chain length
E.
preventing
ligation

Figure 3


plasma membrane
USP6, USP12
intracellular vesicles
endosomes:
USP8, USP2a,
AMSH, AMSH-LP
Golgi apparatus
USP32, USP33,
VCIP135
USP30, USP35 (?)
mitochondria
centrosome
USP21, USP33, CYLD
nucleus
nucleolus
USP36, USP39
USP1, USP3, USP4, USP7,
USP10, USP11, USP12,
USP15, USP16, USP21,
USP22, USP26, USP28,
USP42, USP44, USP49,
USPL1, BAP1, MYSM1,
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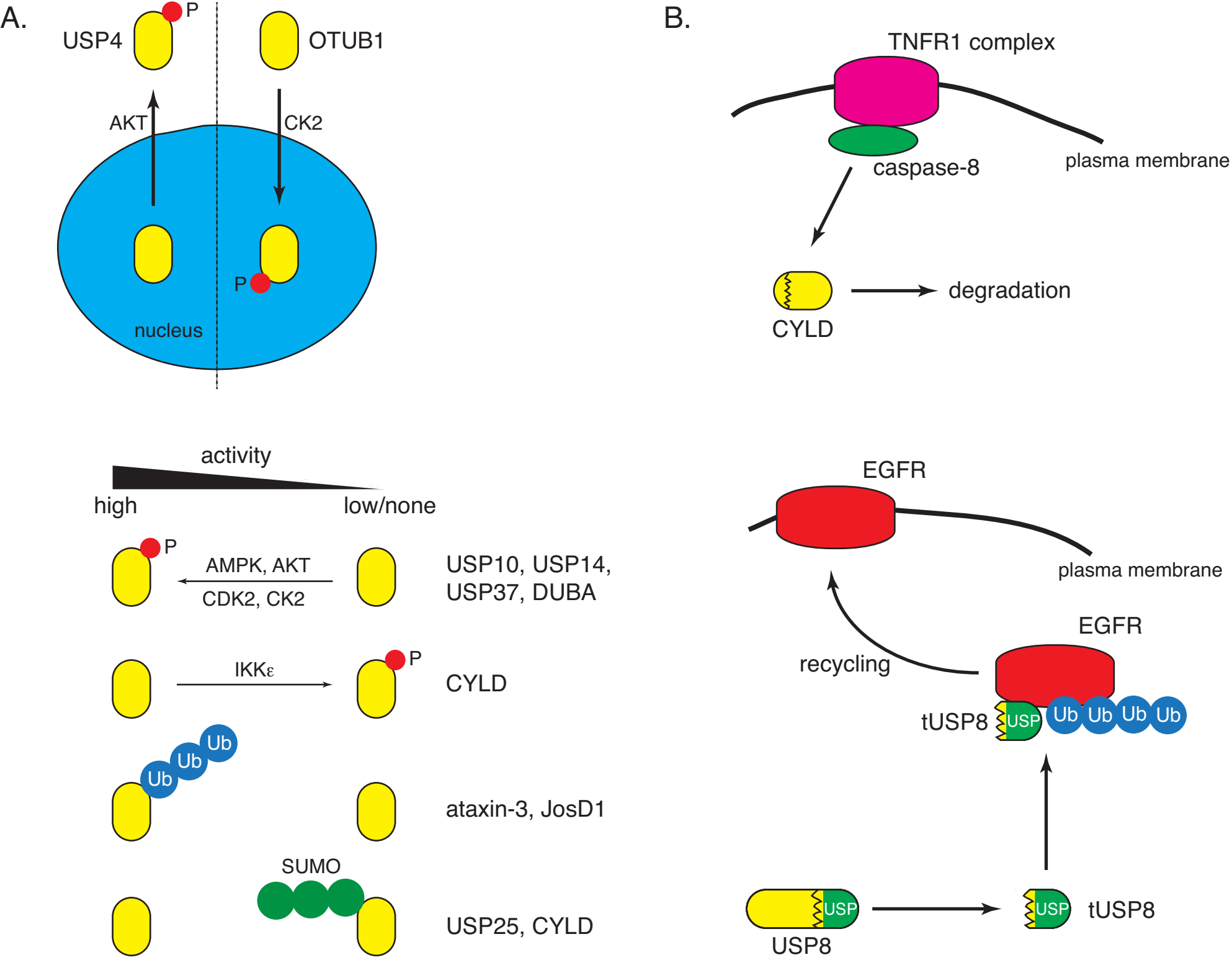
endoplasmic reticulum


A.
USP4
P
OTUB1
AKT
CK2
P
nucleus
activity
high
low/none
P
AMPK, AKT
CDK2, CK2
USP10, USP14,
USP37, DUBA
IKKε
P
CYLD
Ub
Ub
Ub
ataxin-3, JosD1
SUMO
USP25, CYLD
B.
TNFR1 complex
caspase-8
plasma membrane
CYLD
degradation
EGFR
plasma membrane
recycling
EGFR
tUSP8
USP
Ub
Ub
Ub
Ub
USP
USP8
USP
tUSP8

Family	Members	Active
USP	57	51
OTU	16	15
UCH	4	4
MJD	4	4
MINDY	4	4
JAMM	12	9
Total number	**97**	**87**